Effect of purification on the bioavailability of botulinum neurotoxin type A

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Abstract

Botulinum neurotoxins (BoNTs) are among the most potent biological toxins for humans. They are primarily produced by the gram-positive, anaerobic spore-forming bacterium, *Clostridium botulinum*. In bacterial cultures, secreted BoNTs are associated with non-toxic accessory proteins forming large complexes. Neurotoxin-associated proteins have been shown to play an important role in the oral toxicity of BoNTs by protecting them from degradation and digestion by gastric acid and enzymes. Most toxicity studies using BoNTs have been performed using highly purified toxin. In this study, the toxicities of purified and crude BoNT/A toxin preparations were compared. Protein components secreted into culture supernatants along with BoNT/A were identified by mass spectrometry and the contribution of extra proteins found in the soluble crude toxin extracts to the toxicity of BoNTs was determined in mouse models of oral and parenteral botulinum intoxication. Analysis of crude toxin composition permitted assessment of the impact of accessory proteins on the oral bioavailability of BoNT/A toxin in food matrices.

Keywords: Botulinum Neurotoxin type A (BoNT/A), *Clostridium botulinum*, bioavailability, food matrices.

1. Introduction

Botulinum neurotoxins (BoNTs) are recognized as some of the most potent toxins. There are seven known serotypes, A-G, produced by *Clostridium botulinum* as well as a few strains of *C. baratii* and *C. butyricum*. The serotypes most associated with human food-borne infection are types A, B and E (Arnon et al., 2001, Bigalke and Rummel, 2005, Scarlatos et al., 2005). BoNT is synthesized by the bacterium as a ~150 kDa single peptide that is post-translationally processed by bacterial or host proteases into a disulfide-linked dichain toxin containing a ~50 kDa light chain and a ~100 kDa heavy chain protein. The heavy chain contains the translocation (N-terminus region of the heavy chain) and cell-binding domain (C-terminus region of the heavy chain) needed for binding and entry into eukaryotic host cells (Simpson, 2004, Arnon et al., 2001, Singh, 2000). Once internalized in nerves, the light chain containing the catalytic domain cleaves proteins associated with intracellular vesicular transport and consequently inhibits acetylcholine release from neurons, leading to flaccid muscle paralysis.

BoNTs found in nature or secreted into culture media, are usually associated with non-toxic neurotoxin-associated proteins (NAPs) and are also known as “progenitor” toxins. Progenitor toxins can be secreted as complexes of 300, 500 or 900 kDa, also known as M, L, LL toxins of 12S, 16S or 19S molecular sizes respectively (Hines et al., 2005, Inoue et al., 1996, Ohishi et al., 1977). Although NAPs do not play a role in the inhibition of neurotransmitter vesicle docking, they may play a substantial role in oral poisoning by influencing

toxin survival, uptake and transcytosis (Sugii et al., 1977a, Bigalke and Rummel, 2005). Complexed toxins have been shown to be 10 to 100-fold more toxic than non-complexed or “bare” holotoxin through the oral route. This effect has been attributed to the protective effect of the non-toxic protein components of the complexed toxin (Ohishi et al., 1977, Chen et al., 1998). The bioavailability of toxins of varying molecular sizes was investigated using *in vitro* digestion models with pepsin, gastric juices and intestinal juices from rats, and acid (Ohishi et al., 1977, Sugii et al., 1977a, Chen et al., 1998). A direct relationship was observed: the larger the molecular size, the greater the resistance to degradation by pepsin and/or gastric juices. Likewise, the 150 kDa BoNT was more readily digested by pancreatic fluids than were the complexed BoNTs. These studies suggested that the complexed toxin, not the 150 kDa BoNT, causes food-borne botulism (Sugii et al., 1977c, Sugii et al., 1977b, Chen et al., 1998).

Once ingested, BoNTs first encounter the harsh low pH conditions of the stomach and digestive enzymes in small intestines (Sugii et al., 1977a, Maksymowych et al., 1999). Ingested toxins also encounter other protective mechanisms of the intestine like mucins, defensins, trefoil peptides, brush borders, and secretory IgAs (Nagler-Anderson, 2001). After toxin reaches the lumen of the gut, it crosses the intestinal barrier by binding to the apical surface of endothelial cells via receptor-mediated endocytosis. Subsequently, BoNT transcytoses to the basolateral surfaces, reaching the blood and lymph. BoNT is then transported to its target, the peripheral cholinergic nerves, where it inhibits neurotransmitter release (Simpson, 2004).

Certain NAPs, such as the hemagglutinin proteins HA33 and HA17, have been shown to bind epithelial cells, possibly facilitating toxin attachment to the apical surface of the cells and receptor-mediated endocytosis (Simpson, 2004, Fujinaga et al., 2000). NAPs vary among the BoNT serotypes. BoNT A, B, D and G form larger 900 kDa and 500 kDa complexes while BoNT/ E and F form only the smaller 500 KDa and 300 kDa species (Fujita et al., 1995, Hines et al., 2005, Inoue et al., 1996). Little is known about the interaction of BoNT 150 kDa holotoxin with NAPs. Antibody binding studies showed that the non-toxic components of the complex likely interact with the holotoxin through its cell-binding domain (Chen et al., 1997). Furthermore, protease studies on BoNT/D demonstrated interactions between the binding domain of the heavy chain and NTNH and HA70. HA33 and HA17 were exposed to bind epithelial cells, possibly bringing the toxin in close proximity to its receptors (Suzuki et al., 2005).

Though generally thought to be absorbed only from the small intestine, the 150 kDa holotoxin can be absorbed from both the stomach and small intestine (Simpson, 2004, Maksymowych et al., 1999). NAPs, albeit important in ensuring stability of toxin in the intestinal tract, are not absolutely necessary for toxin entry. Strains of *C. botulinum* secreting BoNT/E, which is not complexed with HA, remain toxigenic. Thus, at least two mechanisms for toxin binding and entry may exist: NAP-independent and NAP-dependent. This study explores the contributions of NAPs and other proteins found in crude toxin extracts to BoNT toxicity.

The “gold standard” for the detection and measurement of BoNTs is the intraperitoneal mouse bioassay (Pearce et al., 1997, Schantz and Kautter, 1978b). Few assays approach the mouse bioassay in terms of selectivity and sensitivity; and no *in vitro* assays can fully simulate oral intoxication. Because oral doses to disease are quite high, contamination of food could be detected by using other sensitive non-animal methods such as ELISA (Scarlatos et al., 2005, Sharma and Whiting, 2005, Stanker et al., 2007) however, these do not accurately measure biological activity or account for food matrix effects on toxicity which might give false positives for inactivated toxins (Betley and Sugiyama, 1979, Lindstrom and Korkeala, 2006). Most studies described thus far on the toxicology of BoNTs have been done using highly purified 150 kDa BoNT under standard conditions. Little is known about the properties of crude toxins, the form most likely encountered in intentional food adulteration. In this study, mouse intraperitoneal (ip) or intragastric (ig) models were used to determine the dose-response relationship of purified and crude BoNT/A. The toxicology of purified and crude BoNT/A preparations were compared and their oral bioavailability in food matrices was investigated.

2. Materials and methods

2.1. Materials.

Purified 150 kDa BoNT/A holotoxin and complex BoNT/A were obtained from Metabiologics (Madison, WI) or from the Univ. of Wisconsin and stored at 4 ºC and -20 ºC, respectively. Toxin samples were diluted in phosphate-gelatin buffer (0.028 M sodium phosphate, pH 6.2, 0.2% gelatin). Crude BoNT/A was prepared by buffer extraction of acid mud precipitates of *Clostridium botulinum* Hall A strain at the University of Wisconsin and stored at -20 ºC. Swiss Webster mice (female, 17-23 g) were purchased from Charles River Laboratories (Portage, MI).

2.2 Purified and crude complex toxin analysis.

The concentration of botulinum toxin in purified or crude toxin samples was estimated by SDS polyacrylamide gel electrophoresis (PAGE) using NuPAGE, 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA), followed by densitometry analysis of both Coomassie stained gels and western blots with a FlurChem SP AlphaImager (Alpha Inotech, San Leandro, CA) using known concentrations of BoNT/A holotoxin for standards. Heavy and light chains of the toxin were observed by reducing BoNT/A using 0.1 M 1, 4-dithiothreitol (DTT) at 85 ºC for 10 min. Samples were also analyzed by native PAGE (NativePAGE 3-12% Bis-Tris Gel, Invitrogen).

2.3 Mass spectrometry identification of soluble crude extract components.

Protein spots were excised from gels and then processed by a DigestPro (INTAVIS Bioanalytical Instruments AG, Bergish Gladbach, Germany). Following washing, reduction with DTT, alkylation with iodoacetamide, and in-gel digestion (porcine trypsin, Princeton Separations, Adelphia NJ), the peptides were eluted into a 96 well collection plate with 60 μl of 10% formic acid containing 0.1% trifluoroacetic acid. NanoLC-ESI-MS-MS was done with an Applied Biosystems (ABI/MDS Sciex, Toronto, Canada) Model QStar Pulsar equipped with a Proxeon Biosystems (Odense, Denmark) nano-electrospray source. In-gel digest (20 μl) was loaded automatically onto a C-18 trap cartridge and chromatographed on a reversed-phase column (Vydac 238EV5.07515, 75μ x 150 mm; Hesperia, CA) fitted at the effluent end with a coated spray tip (FS360-50-5-CE, New Objective Inc., Woburn, MA). An LC Packings nano-flow LC system (Dionex, Sunnyvale, CA) with autosampler, column switching device, loading pump, and nano-flow solvent delivery system was used to elute the column. Elution solvents were: A (0.5% acetic acid in water) and B (80% acetonitrile, 0.5% acetic acid). Samples were eluted at 250 nl/min with the following gradient profile: 2% B at 0 min to 80% B in a 15 min linear gradient; held at 80% B for 5 min then back to 2% B for 10 min. The QStar Pulsar was externally calibrated daily and operated above a resolution of 10,000. The acquisition cycle time of 6 s consisted of a single 1s MS "survey" scan followed by a 5 s MS/MS scan. Ions between m/z 400 to 1,000 of charge states between +2 to +5 having intensities greater than 40 counts in the survey scan were

selected for fragmentation. Nitrogen was used for the collision gas and the pressure in the collision cell ranged from 3 x 10^{-6} to 6 x 10^{-6} torr.

The raw data files created by the QSTAR AnalystQS version 1.1 software for each sample were processed by use of a locally installed copy of Mascot Daemon (http://www.matrixscience.com/) and the resulting MS/MS data were analyzed using Mascot (Matrix Science, London, UK; version 2.1.04). Mascot was set to search a local version of a Fasta formatted database of a complete proteome of *C. botulinum* obtained September 2007 from the NCBI. Trypsin was selected as the cleavage enzyme. The results were searched with a fragment ion mass tolerance of 0.2 Da and a parent ion tolerance of 0.2 Da. The iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Methionine oxidation was specified in Mascot as a variable modification. Peptide identifications were accepted if they could be established with probability more significant than 0.05, and protein identifications were accepted if their MOWSE scores were indicative of results more significant than 0.01 and were based on at least 2 identified peptides.

2.4 Food matrices.

For dosing, non-fat milk or whole egg homogenate in phosphate-gelatin buffer was mixed with toxin at a ratio of 5:1 (v/v). Whole eggs were homogenized using an Omni GLH homogenizer (Omni International, Marietta, GA) with a disposable soft tissue tip. Soluble toxin preparations were added to

the food matrices after homogenization. Non-fat milk and whole white chicken eggs (grade A, large size) were obtained from a local retail market and stored at 4 ºC.

2.5 Mouse bioassays.

Groups of 5-10 mice were used at each dosage level and 4 or 5 dose levels were tested per experiment. Randomly grouped mice were inoculated intraperitoneally (ip) with 500 μl or intragastrically (ig by gavage using Popper feeding needles) with 100 μl of sample. Animals were monitored for 7 days for signs of intoxication or death. Mean lethal dose (LD_{50}) values were calculated using the Weil method and/or the Reed and Muench method, depending on the number of doses tested (Weil, 1952, Reed and Muench, 1938). Minimum lethal dose (MLD) was defined as the minimum dose required to kill two mice (Schantz and Kautter, 1978b). Statistical significance using unpaired *t*-tests was determined using the Prism 4 statistics software (GraphPad Software Inc., San Diego, CA). The animal use protocol was approved by the Animal Care and Use Committee of the USDA, Western Regional Research Center, Albany, CA.

2.6 Histopathology of mice exposed to BoNT/A

Mice were dosed with 150 kDa holotoxin, purified or crude complex BoNT/A toxin via ip or ig inoculation routes. Animals were dosed with both lethal (10 or 20 pg of BoNT/A by ip or 0.7 μg of toxin by ig) and sub-lethal doses (5 pg by ip) of pure or crude toxin. Necropsy was performed on selected animals at

times close to death, and on recovering animals that survived challenge (about one week after toxin treatment). Organs were preserved using 10% formalin. Tissue processing, embedding and sectioning were performed at the University of California Davis Center for Comparative Pathology.

3. Results

3.1 Characterization and identification of the components of soluble crude botulinum neurotoxin type A.

During the growth cycle of *C. botulinum*, BoNT holotoxins along with many different proteins are secreted into the surrounding culture medium. Secreted proteins were acid precipitated from the media and subsequently solubilized with buffer to yield a soluble crude toxin extract referred to as “crude” BoNT/A in this study. Further purification of crude toxin by size exclusion purification methods yields large complexes consisting of BoNT and several non-toxic proteins or NAPs (neurotoxin-associated proteins) referred to simply as purified complex BoNT/A in this study. Further purification of BoNT/A from NAPs in the complex, yielded the 150 kDa holotoxin.

Complex BoNT/A was separated by SDS-PAGE under reducing and non-reducing conditions and the NAPs were identified by mass spectrometry. Under reducing conditions, most of the BoNT/A holotoxin in the complex was observed to separate into the heavy and light chains (Fig. 1A). The NAPs identified were: the 130 kDa non-toxic non-hemagglutinin (NTNH) protein which was post-translationally cleaved into the smaller 115 kDa (C-terminus fragment) and 15 kDa fragments (N-terminus fragment), as described by others (Sagane et al., 2002, Fujita et al., 1995); three hemagglutinin proteins HA 33, HA17, and the post-translationally cleaved C- and N-terminal fragments of HA70 (Fig. 1A). Based on densitometry analysis, BoNT/A holotoxin constituted about 30% of the total protein content of the complex. Mass spectrometry also identified a

peptidase of the M20/M25/M40 family (Fig. 1A). It is unclear if this peptidase forms part of the complex or is just loosely associated with the toxin complex, and whether it plays any role in BoNT/A toxicity.

When compared with purified holotoxin (150 kDa) or purified complex BoNT/A (complex), the soluble crude extract (crude) contained several additional proteins that were not found or that were less abundant in the purified complex (Fig. 1B, see arrows). These proteins (labeled 1-10) were excised from the SDS-PAGE and analyzed by mass spectrometry (Fig. 1B and Supplemental table 1). Proteins not present in the purified complex include: NAD-specific glutamate dehydrogenase, ornithine carbamoyltransferase, phosphate ABC transporter protein, nlpc/p60 family protein, ATP dependent Clp protease, endoribonuclease, oxidoreductase, thiolase, the C-terminus of a carbohydrate binding protein and a single strand binding protein. Proteins that were found to be less abundant in the purified complex included: HA70, HA33 and NTNH (either intact or truncated fragments). Interestingly, we also identified a C-terminal peptide fragment of a bacterial immunoglobulin-like domain protein (Big) associated with BoNT/A and NTNH, forming a protein complex that appears as a single band at about 180 kDa (Fig. 1B).

3.2 Native PAGE of purified and crude BoNT/A toxin.

Purified BoNT/A complex and crude BoNT/A were analyzed by native PAGE to determine their NAP composition and size of their complexes (Figure 2 and Supplemental Table 2). Pure 150 kDa holotoxin formed multimers that

migrated in a ladder-like pattern. High molecular weight species that migrated in the region between the 720 and 1048 kDa markers predominated in both preparations (bands 1-3). Mass spectrometry indicated that Band 1 contained BoNT/A, HA70, NTNH, HA33, and HA17. Bands 2 and 3 were qualitatively similar, but had different ratios of components. In addition, components were identified that were unique to either purified (band 5) or crude (band 6) toxin complexes. Band 6, a prominent component of crude toxin, contained BoNT/A, HA33, and NTNH. Crude toxin bands (Supplemental table 2, bands 1, 2, 3, 4, 6 and 7) contained additional components, such as the M20/M25/M40 family peptidase, Big domain protein, phosphate ABC transporter, and ornithine carbamoyltransferase. These additional proteins are probably loosely associated in unspecific interactions with the BoNT/A complex because they can be removed by further purification steps. As observed by SDS-PAGE, the major difference between purified and crude toxin preparations is that of the NAPs.

3.3 Mouse models for BoNT/A intoxication

We tested the toxicity of purified complex and crude BoNT/A toxins using the mouse intraperitoneal (ip) and intragastric (ig) models. Mice treated by ip injection showed most of the classic symptoms of botulism: ruffled fur, “wasp-waist”, hind limb paralysis (difficulty of movement), labored breathing, and death (Schantz and Kautter, 1978a, Scarlatos et al., 2005). Mice treated by ig injection did not consistently show prominent wasp-waists.

We next investigated the effect of NAPs on the mean lethal dose (LD_{50}) in the ip model. As shown in Table 1, on the basis of BoNT/A content, the purified complex and crude BoNT/A had LD_{50}s of 0.43 and 0.42 ng/kg, respectively, about 8-9 pg/mouse, similar to that of the 150 kDa holotoxin. Although the associated proteins in the purified complex and crude BoNT/A did not appear to influence BoNT/A LD_{50} ip, further analysis of the dose-response data revealed subtle differences in kinetics (Figs. 3A and 3B). At high dosage (20 pg/mouse, squares), the 150 kDa holotoxin caused more rapid lethality than did crude toxin extracts. At lower dosages (e.g., 5 pg/mouse, filled circles), crude toxin extracts had the more rapid effect.

BoNT/A ig toxicity was next explored using oral gavage. As shown in Table 1, the 150 kDa holotoxin MLD was 1 x 10^5 times lower in toxicity by ig compared to ip, a factor similar to that reported in previous studies (Ohishi et al., 1977, Sugiyama et al., 1974). The toxicities of different forms of the toxin were explored using minimal lethal dose (MLD). The MLD for holotoxin was 12 µg, 17-fold greater that the MLD for both the purified and crude complexes of BoNT/A (Table 1). Thus, the NAPs enhanced oral toxicity, whereas the loosely associated proteins in the crude extract did not appear to significantly affect oral toxicity.

3.4 Bioavailability of pure and crude BoNT/A in food matrices

The LD_{50} of the purified complex and crude BoNT/A were evaluated in two food matrices: non-fat milk and homogenized whole egg (Table 2 and Fig 4A).

Purified complex BoNT/A administered ig in buffer, milk or egg did not show any significant difference in toxicity. Toxicity in non-fat milk was slightly lower but not statistically significant (p = 0.10 as determined by the unpaired t-test). Crude BoNT/A had similar toxicity to purified complex in buffer or egg matrices (Table 2). In the non-fat milk matrix, the LD_{50} of crude toxin was 65 μg/kg, a two-fold decrease in toxicity compared to the LD_{50} of 32 μg/kg in the buffer matrix (p = 0.0067, Table 2 and Fig. 4B). This change in toxicity was route-dependent. When crude toxin was administered to mice ip in the non-fat milk matrix, toxicity was not significantly different than that observed in the buffer matrix (data not shown). Survival of mice treated with purified complex versus crude BoNT/A in the non-fat milk matrix consistently showed a slower die-off for crude BoNT/A treated mice (Fig 4 C and 4D).

The BoNT/A (150 kDa) content in various preparations of crude toxin ranged from 15-24% of total protein as determined by densitometry analysis of Coomassie stained gels and western blots (data not shown). To determine LD_{50}, the amount of crude toxin was normalized by BoNT/A content. Thus, samples spiked with extracts of low BoNT/A content had more extraneous proteins added, compared to high BoNT/A content preparations. We next wanted to determine whether quantity of extraneous proteins had any observable effect on toxicity (e.g., a toxin protecting effect in the oral model). The LD_{50} of crude extracts with different percentages of BoNT/A in total protein (15, 17, 20 and 24%) were compared to the LD_{50} of purified complex (where about 30% of total protein content is BoNT/A) for both ip and ig dosing. No observable difference in toxicity

was observed following ip exposure (data not shown). A small, but not significant change in LD_{50} was observed following oral exposure (Fig. 5).

3.5 Histopathology of mice subjected to BoNT/A intoxication

Mice injected with high or low doses of pure or crude BoNT/A toxin were examined for histopathological lesions. Necropsy was performed on mice with the terminal symptoms of fatal botulism (labored breathing and paralysis). The most notable histopathological changes in these mice were increased lymphocytic apoptosis in the thymus and moderate or marked lymphoid reactivity in the spleen and lymph nodes. In the thymus, there were moderate cortical lymphocytic apoptosis associated with tingible body macrophages and minimal apoptosis in the medulla. In the spleen and lymph nodes, there was mild to moderate lymphoid hyperplasia and many follicles had prominent germinal centers which was consistent with up-regulated lymphoid reactivity. Mice that were recovering from non-lethal botulism showed moderate lymphoid reactivity in the spleen and lymph nodes about a week after intoxication. No notable differences in pathology were observed among mice treated with the 150 kDa holotoxin, purified complex or the crude toxin. There were also no histological differences between mice receiving ip vs. ig exposure to toxin challenge.

4. Discussion

This study sought to characterize crude BoNT/A extracts with regard to protein composition, toxicity, and bioavailability in food matrices. First, proteins identified in the soluble crude extracts of BoNT/A but not present, or markedly reduced in abundance in purified complex BoNT/A, were identified using mass spectrometry analysis of SDS-PAGE and native gels (Fig. 1B and Fig. 2 and Supplemental Tables 1 and 2). The most striking features in these toxin preparations were the presence of excess NAPs, including NTNH, HA70 and HA33. Since NAPs are thought to play a role in protecting toxin from degradation, epithelial cell binding, and toxin entry (Simpson, 2004), the presence of additional NAPs could potentially have an effect on toxicity or bioavailability.

No difference in toxicity for the 150 kDa holotoxin, purified complex, and crude BoNT/A preparations administered by ip, when protein concentrations were adjusted for BoNT/A content (Table 1). NAPs or extraneous proteins found in the crude extracts did not have any apparent effect on toxicity. This observation could be explained by the disassembly of complexes *in vivo* at the mildly alkaline physiological pH of blood and HA binding to blood components (Simpson, 2004, Chen et al., 1997). Although LD_{50}'s were similar for pure and crude toxins, the kinetics of killing differed slightly (Fig. 3 A and B). Although the 150 kDa holotoxin killed faster than crude toxin at high doses, faster killing was observed for the crude toxin at low doses. Our observations agree with previous research that demonstrated that lower molecular weight 150 kDa holotoxin killed

mice more rapidly than larger molecular sized complex toxin (Lamanna et al., 1970, Ohishi et al., 1977, Sugii et al., 1977b). The time-to-death response could be dependent on the conversion of larger molecules to smaller ones or on the uptake or transfer of different sized molecules to the target cells (Lamanna et al., 1970). The larger complexed BoNT/A may be more stable and therefore retain its toxicity at low doses better than the holotoxin.

In agreement with previous studies, the oral toxicity of complex BoNT/A was significantly greater than that observed for the holotoxin (Ohishi et al., 1977, Sugiyama et al., 1974). The BoNT/A required for lethality is reduced by about 17 times when it is complexed with other proteins (Table 1). No statistically significant difference in the LD_{50} was observed in mice dosed orally with purified complexes in buffer or food matrices (Table 2 and Fig. 4 A & B). Purified complex and crude toxins had similar toxicities in both buffer and egg matrices. However, the toxicity of crude BoNT/A decreased two-fold when administered in the non-fat milk matrix (Table 2 and Fig. 4A). This effect of the non-fat milk matrix was observed only during ig but not ip dosing (data not shown).

A variety of factors may have contributed to the reduced toxicity of crude toxin in the non-fat milk matrix. Proteins such as HA 33 and HA17 have been shown to bind to epithelial cells, and the presence of additional HA 33 or other NAPs may compete with the toxin complex for entry (Niwa et al., 2007, Fujinaga et al., 2000). However, crude BoNT/A was not significantly different in toxicity from purified complex BoNT/A in the buffer matrix. Thus, any contribution of excess NAPs depended on the presence of the non-fat milk matrix. In contrast to

these results, the toxicity of purified complex BoNT/A did not change significantly in the non-fat milk matrix. Therefore, in the absence of excess NAPs, milk alone does not change toxicity. A recent study on the absorption of BoNT/B showed that human secretory IgA binds to BoNT/B and limits its binding to cultured cells (Matsumura et al., 2007a). Because secretory IgA is the major immunoglobulin found in gastrointestinal secretions, neutralization of microbes and toxins and subsequent inhibition of toxin binding to epithelial cells might occur. Toxin binding to HA proteins could also be inhibited by galactose or other sugars present in non-fat milk (Matsumura et al., 2007a, Fujinaga et al., 2000). We hypothesize that a component of non-fat milk interacts with NAPs in crude BoNT/A to reduce bioavailability. Because different crude toxin preparations vary in their content of NAPs, we expected to see an impact on toxicity. However, a consistent trend was not observed. Moreover, ip toxicity did not correlate well with ig toxicity among the different preparations. Although a simple explanation for these observations is lacking, the results indicate that time-to-death following ip exposure, a commonly used bioassay, may not yield a consistent measure of toxin content or oral toxicity for complexed BoNTs.

Previous researchers have shown that purified 150 kDa holotoxin can cross the epithelial cell barrier by transcytosis and such toxin can also can bind selectively to intestinal epithelial cells via surface glycoconjugates, surface receptors such as gangliosides and specific protein receptors for BoNT/A such as SV2 (Matsumura et al., 2007a, Matsumura et al., 2007b, Dong et al., 2006). These studies, taken together with the present data, suggest that BoNT/A entry

can occur by both NAP-dependent and independent mechanisms. Our mass spectrometry data also revealed an intriguing association between BoNT/A and bacterial Immunoglobulin-like domains proteins (Big). Big protein domains have been shown to bind carbohydrate residues and are though to play a role in cell binding. These domains exist in bacterial proteins such *Yersinia* invasins and *E. coli* intimins (Dersch and Isberg, 2000, Fraser et al., 2006). They are also present in bacteriophages that infect both gram-positive and gram-negative bacteria, and may aid in the attachment of the phage particles to the host cell surface. The co-migration of Big protein domain with BoNT/A in SDS-PAGE suggests the existence of an alternate cell-binding or entry pathway. Other protein components of the crude toxin extracts, such as the carbohydrate binding proteins, may also contribute to toxicity. Although not apparent in this study, their influence may depend on food matrix components or host systems.

Although absorption, translocation, and toxicity of BoNTs differ by the route of exposure, intoxication by all routes results in paralysis of muscles, especially those involved in respiration (such as the diaphragm) leading to fatal asphyxiation (Arnon et al., 2001). Histopathological comparison of mice treated ip or ig showed moderate lymphoid reactivity in the spleen and lymph nodes and mice that received lethal doses of toxin also had increased rates of lymphocytic apoptosis in the thymus. The histological changes were for both pure and crude BoNT/A preparations. Although not likely the cause of death in mice, immunopathology may be an additional effect of botulinum intoxication that was previously unrecognized. The impact of BoNT/A intoxication on disease or

infection susceptibility warrants further study. The histopathology results also suggest that early immune responses could serve as biomarkers of BoNT intoxication. The potential for early screening for BoNT intoxication could lead to earlier treatment, reduced hospitalization time and more rapid recovery.

There are fewer than 200 reported cases of human botulism per year in the U.S. Of these, most are infant and wound botulism cases and only a few are food-borne. Although food-borne botulism is not a prominent problem, the extreme toxicity, relative ease of strain acquisition and toxin production, make adulteration of food sources with BoNT a potential bioterrorism concern (Scarlatos et al., 2005, Arnon et al., 2001, Bigalke and Rummel, 2005). Recent mathematical modeling of possible bioterrorist attacks on a food source showed how small quantities of toxin could cause large-scale human exposure and economic devastation (Wein and Liu, 2005). Bioterrorist attacks on food sources would likely involve crudely prepared toxin materials. Thus, a better understanding of the toxin, its mode of contamination in food, and its bioavailability in complex food matrices would help prepare emergency personnel for potential attacks.

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Supplemental Table 1

Mass spectrometry identification of soluble crude BoNT/A protein bands separated on SDS-PAGE (Fig. 1B). Proteins listed were either not present in purified complex samples or reduced in abundance. RefSeq accessions appear in parenthesis.

Protein band	Proteins present
1	BoNT/A (YP_001386738.1), bacterial immunoglobulin-like domain (YP_001387531.1), NTNH (YP_001386737.1)
2	Peptidase of the M20/M255/M40 family (YP_001388317.1), HA70 (YP_001386733.1), ornithine carbamoyltransferase (YP_001388305.1), HA33 (YP_001386735.1), BoNT/A
3	HA70 (C-term.), glutamate dehydrogenase (YP_001387608.1), ornithine carbamoyltransferase, HA33
4	Ornithine carbamoyltransferase, glyceraldehyde-3P dehydrogenase (YP_001386182.1), HA70, HA33
5	HA33, ornithine carbamoyltransferase, phosphate ABC transporter (YP_001388227.1), HA70 (C-term.)
6	HA33, phosphate ABC transporter, ornithine carbamoyltransferase , triosephosphate isomerase (YP_001386184.1), oxidoreductase (YP_001389155.1), aldo/keto reductase family (YP_001389094.1)
7	NlpC/P60 family protein (YP_001387668.1), ornithine carbamoyltransferase, rubrerythrin (YP_001387227.1), HA33, glutamate dehydrogenase, ATP-dependent Clp protease, proteolytic subunit ClpP (YP_001388961.1), thiolase (YP_001388929.1)
8	HA 70 (N-term.), carbohydrate binding protein (C-term.) (YP_001387758.1), ornithine carbamoyltransferase, ferritin family protein (YP_001387583.1)
9	HA17 (YP_001386734.1), methionyl-tRNA synthetase (YP_001386034.1) (C-term.), HA70 (N-term.), carbohydrate binding protein (C-term.), thiolase, desulfoferrodoxin (YP_001389058.1), V-type ATPase, K subunit (YP_001388342.1)
10	Putative endoribonuclease L-PSP (YP_001388709.1), oxidoreductase, single-strand binding protein (YP_001389348.1), hypothetical protein CLC_2446 (YP_001388287.1), NTNH (N-term.), HA17 (N-term.)

Supplemental Table 2

Mass spectrometry identification of purified complex BoNT/A and soluble crude BoNT/A protein bands separated on native gel (Fig. 2). Proteins were listed in descending order of abundance as determined by mass spectrometry. Bands 4 and 6 appear in crude BoNT/A but not present or not abundant in the purified complex.

Protein band	Purified complex BoNT/A	Crude BoNT/A
1	BoNT/A, NTNH, HA70, HA33, HA17	BoNT/A, NTNH, HA70, HA33, HA17
2	NTNH, HA70, HA33, BoNT/A, HA17	NTNH, HA70, HA33, BoNT/A, HA17
3	HA70, NTNH, BoNT/A, HA33, HA17	HA70, NTNH, BoNT/A, peptidase of M20/M255/M40 family, HA33, HA17, bacterial immunoglobulin-like domain protein
4		BoNT/A, ornithine carbamoyltransferase, phosphate ABC transporter, HA33, bacterial immunoglobulin-like domain protein, HA70, NTNH
5	BoNT/A, NTNH, HA70, HA33	
6		Ornithine carbamoyltransferase, BoNT/A, HA33, NTNH
7	NTNH	NTNH, HA33, endoribonuclease, ornithine carbamoyltransferase, phosphate ABC transporter, BoNT/A

Table 1

Intraperitoneal LD_{50} and oral lethality of botulinum toxin preparations.

BoNT/A[a]	ip LD_{50} (ng/kg)	ig MLD[b] (μg/mouse)
150 kDa	0.42 ± 0.02	12
Complex	0.43 ± 0.05	0.7
Crude	0.42 ± 0.04	0.7

[a] Concentration of BoNT/A in purified complex or in crude BoNT/A were estimated by densitometry following SDS-PAGE

[b] MLD: minimum lethal dose

Table 2

Oral LD_{50} of purified complex and crude BoNT/A[a].

Food matrix	LD_{50} (μg/kg)[b]	
	Complex	Crude
Buffer	27 ± 3	32 ± 4
Non fat milk	31 ± 4	65 ± 8
Whole egg	28 ± 4	29 ± 2

[a] BoNT/A content in purified complex or crude samples was determined using densitometry analysis

[b] Mean ± SEM.

Figure Legends

Fig. 1. A. SDS-PAGE of purified complex BoNT/A under reducing and non-reducing conditions. Proteins were separated on 4-12% Bis-Tris gel. Sample on the left was reduced with DTT to separate heavy and light chains of the toxin. B. SDS-PAGE of purified BoNT/A holotoxin (150 kDa) purified complex BoNT/A (Complex) and soluble crude BoNT/A toxin (Crude). Crude BoNT/A was prepared from buffer extraction of acid precipitated *C. botulinum* culture supernatants. Identities of protein bands were confirmed by mass spectrometry analysis. MW: molecular weight marker. Components of protein bands in B were listed in Supplemental table 1.

Fig. 2. Native protein electrophoresis of purified and crude BoNT/A. Proteins were separated on a NativePage 3-12% Bis-Tris Gel (Invitrogen). Components of bands (1-7) were identified by mass spectrometry analysis and listed in Supplemental table 2.

Fig. 3. Survival curves of mice treated with 150 kDa BoNT/A holotoxin (A) or crude BoNT/A (B). Percent survival was plotted over time. Mice were dosed as follows: 20 pg/mouse (squares), 10 pg/mouse (triangles), 5 pg/mouse (circles), and 2.5 pg/mouse (diamonds).

Fig. 4. Bioavailability of purified and crude BoNT/A in food matrices. The LD_{50} of purified complex and crude BoNT/A in liquid matrices of phosphate gelatin (pH.

6.2), non-fat milk and liquid whole egg were plotted in diagrams A and B respectively. Survival curves of mice challenged ig with different doses of purified complex BoNT/A (C) and crude BoNT/A (D) in the non-fat milk matrix were plotted.

Fig. 5. Bioavailability of crude BoNT/A in food matrices. BoNT/A content in soluble crude toxin preparations ranged from 15-24% of the total protein content. Upon further purification, BoNT/A comprised about 30% of the total protein content in the purified complex.

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